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α7 Nicotinic Acetylcholine Receptor Regulates Airway Epithelium Differentiation by Controlling Basal Cell Proliferation

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Airway epithelial basal cells are known to be critical for regenerating injured epithelium and maintaining tissue homeostasis. Recent evidence suggests that the α7 nicotinic acetylcholine receptor (nAChR), which is highly permeable to Ca^{2+}, is involved in lung morphogenesis. Here, we have investigated the potential role of the α7 nAChR in the regulation of airway epithelial basal cell proliferation and the differentiation of the human airway epithelium. *In vivo* during fetal development and *in vitro* during the regeneration of the human airway epithelium, α7 nAChR expression coincides with epithelium differentiation. Inactivating α7 nAChR function *in vitro* increases cell proliferation during the initial steps of the epithelium regeneration, leading to epithelial alterations such as basal cell hyperplasia and squamous metaplasia, remodeling observed in many bronchopulmonary diseases. The regeneration of the airway epithelium after injury in $\alpha7^{-/-}$ mice is delayed and characterized by a transient hyperplasia of basal cells. Moreover, 1-year-old $\alpha7^{-/-}$ mice more frequently present basal cells hyperplasia. Modulating nAChR function or expression shows that only α7 nAChR, as opposed to heteropentameric $\alpha_x\beta_y$ nAChRs, controls the proliferation of human airway epithelial basal cells. These findings suggest that α7 nAChR is a key regulator of the plasticity of the human airway epithelium by controlling basal cell proliferation and differentiation pathway and is involved in airway remodeling during bronchopulmonary diseases. ***(Am J Pathol 2009, 175:1868–1882; DOI: 10.2353/ajpath.2009.090212)***

The respiratory epithelium, which is constantly exposed to airborne pollutants, is frequently injured, which results in altered epithelial functions. To restore these functions, the respiratory epithelium must undergo rapid repair via epithelial cell spreading and migration and regenerate its structure via basal cell proliferation and differentiation.[1] These processes are tightly controlled to restore the pseudostratified architecture of the normal mucociliary epithelium. However, in most respiratory diseases, alterations of the regeneration processes induce epithelial remodeling such as hyperplasia, metaplasia, and fibrosis. Understanding the sequence of processes involved in cell proliferation and differentiation is therefore of crucial importance. Both *in vivo* and *in vitro*, human airway basal cells are able to proliferate and reconstitute a fully differentiated and functional epithelium.[2] These cells are such considered as progenitors of the human airway epithelium and important actors of the airway epithelium regeneration.

The nonneuronal cholinergic system is thought to be involved in the regulation of cell functions such as cell-cell interaction, apoptosis, and proliferation.[3] It is now established that human bronchial epithelial cells contain all of the machinery for the production, storage, secretion, and degradation of acetylcholine, which acts as an autocrine or paracrine hormone.[4,5] Acetylcholine exerts its effects through muscarinic and nicotinic acetylcholine receptors. Nicotinic acetylcholine receptors (nAChRs)

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are composed of five subunits, arranged as α/β heteromeric or α homomeric nAChRs, and assembled around a central ion channel, mediating the influx of Ca^{2+}.[6] The airway epithelium expresses α3, α4, α5, α7, α9, β2, and β4 subunits for nAChRs.[7–9]

α7 nAChR is characterized by an elevated Ca^{2+} permeability[10] and has been involved in several important biological processes such as cell proliferation, apoptosis, and angiogenesis in cancer.[11,12] Prenatal nicotine exposure significantly increases pulmonary α7 nAChR expression and alters fetal lung development[13] and subsequently pulmonary function in newborn.[14] In particular, alteration of lung branching morphogenesis induced by nicotine is mediated by α7 nAChR.[15] Altogether, these observations led us to investigate whether the α7 nAChR could be involved in the differentiation of the respiratory epithelium. In the human airway epithelium, we observed α7 nAChR expression in basal cells, which play a critical role in the epithelial regeneration. Both *in vivo* and *in vitro*, the α7 nAChR expression is associated with the airway epithelium differentiation. Moreover, *in vitro* inactivating α7 nAChR or *in vivo* disrupting genetic α7 nAChR expression induces airway epithelium remodeling by modulating basal cell proliferation. This study thus provides several lines of evidence that α7 nAChR is significant for airway epithelial differentiation and suggests that α7 nAChR is a key regulator of the plasticity of the airway epithelium.

Materials and Methods

Reagents

Unless otherwise stated, all reagents were from Sigma-Aldrich Chimie (L'Isle d'Abeau Chesnes, France): mouse anti-cytokeratin (CK)-13 (clone KS-1A3; 1/1000), mouse anti-CK14 (clone LL02; 0.7 μg/ml), mouse anti-Ki-67 (5 μg/ml) (Dako, Trappes, France), mouse anti-β-tubulin (clone KMX-1; 0.1 μg/ml) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (MAB374; 1 μg/ml) (Millipore, St. Quentin en Yvelines, France), mouse anti-human cystic fibrosis transmembrane conductance regulator (clone 24-1; 2 μg/ml) (R&D Systems, Minneapolis, MN), mouse anti-zonula occludens (ZO)-1 (clone ZO1-1A12; 1/20) (Zymed Laboratories, San Francisco, CA), mouse anti-desmoplakin 1-2 (clone DP1 and 2-2.15; 20 μg/ml) (Harlan Sera-Lab, Loughburough, UK), rabbit polyclonal antibodies anti-nAChR α7 (H-302, lot A2203; 1 μg/ml), anti-caveolin-1 (N-20; 2 μg/ml) and anti-α-ENaC (H-95; 1 μg/ml) and mouse anti-Foxj1 (clone 3–19; 2 μg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-transglutaminase-1 (0.2 μg/ml) (Covalab, Cambridge, UK), 4′,6′-diamino-2-phenylindole (200 ng/ml), *Griffonia simplicifolia* isolectin B_4-FITC (GSI-B4; 4 μg/ml), Alexa Fluor 488 and Alexa Fluor 594 goat anti-mouse or anti-rabbit (IgG H+L; 1/200) secondary antibodies, αBTX-Alexa Fluor 488 conjugate, rabbit anti-Alexa Fluor 488 (5 μg/ml) and donkey anti-rabbit IgG-Alexa Fluor conjugate (10 μg/ml) (Molecular Probes, Eugene, OR), and α-conotoxin MII was synthesized by Geneprep (Montpelier, France) according to Ref.[16]

Human Airway Tissues and Ex Vivo Wound Repair Model

The use of human tissues was authorized by the bioethical law 94-654 of the Public Health Code of France, with a written consent from the patients or their families. Tracheas from normal human fetuses ranging from 10 to 25 weeks of development were obtained after medically induced abortions. Human airway tissues were collected after nasal polypectomy of patients who did not suffer from any other disease. Human bronchial tissues from patients undergoing surgery for bronchial carcinoma were obtained from microscopically normal areas distant from the tumor.

Human bronchial tissue samples were locally injured and the repair process was followed for 4 days as described previously.[17,18]

Epithelial Cell Isolation and Culture

Human airway epithelial cells (HAECs) were isolated from polyps and bronchial tissues and cultured in an air-liquid interface culture condition as described previously.[2,19,20] After 5, 7, 9, 11, 16, 23, 30, and 33 days, cells were detached with trypsin and counted with the ADAM-MC apparatus (Labtech, France).

Human airway basal epithelial cells were sorted and separated from secretory and ciliated cells by FACS as described previously.[2] The sorted cells were composed of 98.4% basal cells as demonstrated by the reanalysis of the sorted cells.

Immunocyto/histochemistry

An indirect immunofluorescence labeling technique was performed on frozen sections of bronchial tissues or cell cultures as described previously.[19] All fluorescence-labeled preparations were examined with an AxioImager microscope (Zeiss, Oberkochen, Germany) equipped with a Coolsnap FX camera (Roper Scientific, Lisses, France). Images were processed with the AxioVision (Zeiss) and Photoshop (Adobe Systems, San Jose, CA) software.

Localization of Binding Sites for α-Bungarotoxin

Binding sites for α-bungarotoxin (αBTX) were studied in bronchial tissue samples according to Ref.[21] with some modifications, including the following steps: 1) overnight incubation at 4°C with 1 μmol/L αBTX-Alexa Fluor 488 conjugate, 2) fixation with 4% paraformaldehyde for 20 minutes at room temperature, 3) overnight incubation at 4°C with 5 μg/ml rabbit anti-Alexa Fluor 488, 4) incubation for 1 hour at room temperature with 10 μg/ml donkey anti-rabbit IgG-Alexa Fluor 488 conjugate, and 5) counterstaining with hematoxylin. Controls were performed by omitting αBTX-Alexa Fluor 488 conjugate in the first incubation.

Cell Proliferation Assay

Freshly isolated HAECs or FACS sorted basal epithelial cells were seeded on 96-well microplates in a proliferation medium, adapted from Refs.[19] and [22] at a density of 20×10^3 cells/cm^2, and cultured in the presence of 0–10 μmol/L αBTX, methyllycaconitine, α-connotoxin MII, or mecamylamine. Cells were then incubated for 1 hour in 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide. The dye was extracted with propanol-2 and the OD at 560 nm was read in a Xenius spectrophotometer (Safas, Monaco).

Transfection of Small Interfering RNA and nAChR α7 Expression Vector and RT-PCR Analyses

Two α7 subunit-specific 19-nt sequences were selected in the coding sequence of nAChR α7 subunit (GenBank accession no. NM000746) to generate 21-nt sense and 21-nt antisense strands of the type (19N) TT (N, any nucleotide). The selected 19-nt sequences were as follows: α7si1, 5′-CAUCCGAUUCGGUACCAUU-3′; and α7si2, 5′-GGACAGAUCACUAUUUACA-3′ (Eurogentec, Seraing, Belgium). Two duplexes, which do not recognize any sequence of human genome, were used as control. A total of 20,000 16HBE14o-cells were transiently transfected with 20 nmol/L siRNA duplexes by using the calcium phosphate-precipitation method. Twenty-four hours after transfection, cells were counted and collected for RT-PCR analysis.

Using FuGENE 6 reagent (Roche Diagnostics, Meylan, France), 20,000 16HBE14o-cells were transiently transfected with 1 μg/ml pCMV6 empty vector or 1 μg/ml pCMV6-cDNA α7 (OriGene, Rockville, MD), according to the manufacturer's procedure. The cells were counted and collected for RT-PCR analysis at 1, 3, and 6 days after transfection.

RT-PCR analyses were performed as previously described,[23] using primers for human α7 nAChR (5′-CAGTCTTACTCTCTCTTACCGTCT-3′ and 5′-GCACCAGTTCAGAAGGATGACTC-3′; Eurogentec).

Western Blotting

Quantification of α7 nAChR by Western blotting was performed as previously described,[23] using the H-302 anti-α7 nAChR antibody. Subsequent detection of glyceraldehyde-3-phosphate dehydrogenase was performed on the same filters as a control.

Transgenic Mice

All experiments and procedures were performed in compliance with the French Ministry of Agriculture regulations for animal experimentation. Mice lacking the α7 subunit of the nicotinic receptor (C57BL/6 background) and wild-type littermates were generated as previously reported,[24] shipped from Institut Pasteur (Paris, France) and housed in a sterile animal care facility. Mice, used for the study of the tracheal epithelium regeneration process, were ~8 weeks of age. The phenotype of tracheal epithelium was also investigated in 1-year-old mice.

Induction of Tracheal Epithelial Damage in Mice

Epithelial desquamation was induced in mouse trachea after instillation of polidocanol.[25] Thirty-five microliters of 2% polidocanol in PBS was slowly instilled into the nostrils. Animals were sacrificed on days 0 (before polydocanol treatment), 2, 4, 6, 7, and 8, and trachea was removed. The morphology of the tracheal epithelium was studied in 10 hematoxylin-stained sections covering the entire proximal region of the trachea extending 1–3 mm beneath the larynx. The epithelial height was measured in three different areas of the tracheal epithelium.

Characterization of the Tracheal Epithelium Histology in $\alpha7^{+/+}$ and $\alpha7^{-/-}$ 1-Year-Old Mice

The morphology of the tracheal epithelium was studied in $\alpha7^{+/+}$ and $\alpha7^{-/-}$ 1-year-old mice, in ~10–15 hematoxylin-stained sections covering the entire proximal region of the trachea extending 1–3 mm beneath the larynx. In all sections derived from $\alpha7^{+/+}$ and $\alpha7^{-/-}$ mice, three different phenotypes of the surface epithelium were observed. In each section, we assessed the percentage of each phenotype along the total length of the tracheal epithelium covering cartilage.

Results

α7 nAChR Expression Is Associated with the Differentiation of the Airway Epithelium

When using the H-302 polyclonal antibody or αBTX, a competitive antagonist with a high affinity for the α7 nAChR,[10,26,27] we localized the α7 nAChR in the normal human airway epithelium, both at the apical plasma membrane of columnar ciliated cells and in the pericellular plasma membrane of basal cells (Figure 1, A and B).

To study the relationship between α7 nAChR expression and the differentiation of the airway epithelium, we studied the expression of α7 nAChR both *in vivo* in human tracheal tissue samples during fetal development and *in vitro* during the regeneration of human airway epithelium. The human fetal airway epithelium acquires its morphological differentiation at ~12 to 15 weeks of development.[28] We observed that, during the fetal development of human airways, α7 nAChR expression starts at 13 weeks of development, simultaneously with the expression of ZO-1, a marker of epithelial cohesion, of β-tubulin, a marker of the ciliary differentiation and of cystic fibrosis transmembrane conductance regulator, a marker of epithelium functionality for Cl^- ion secretion at the apical membrane (Figure 2). The human airway epithelium can be fully regenerated *in vitro* from isolated HAECs maintained in culture in an air-liquid interface condition.[20] In

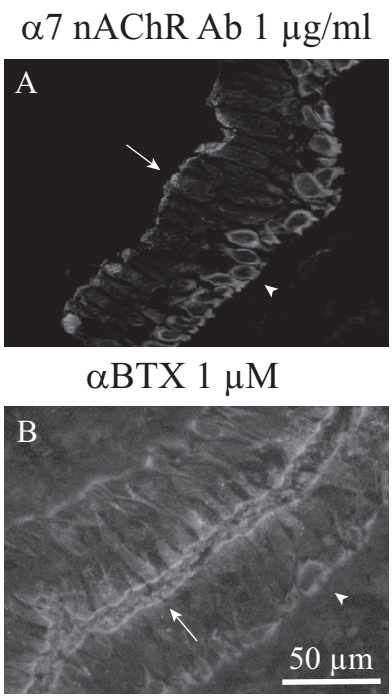

Figure 1. Localization of nAChR α7 in the airway epithelium. nAChR α7 was localized with the H-302 antibody at 1 μg/ml (**A**) or with AlexaFluor488-αBTX at 1 μmol/L (**B**) in human bronchial tissue samples, both at the apical side of the epithelium (**arrows**) and in basal epithelial cells (**arrowheads**).

this model, we observed a progressive regeneration of pseudostratified epithelium after 4 to 6 weeks (Figure 3A). By using immunofluorescence and RT-PCR techniques, we observed that the expression of α7 nAChR in the basal layers of the epithelium was maximal in steps II and III (appearance of the first ciliated cells) and paralleled the expression of CK14, a marker of airway basal cells,[29] suggesting that α7 nAChR expression in basal cells is essential during the initial step of epithelial differentiation and the establishment of the basal cell layer. Thereafter, the expression became progressively restricted to one single layer of basal cells for α7 nAChR or isolated basal cells for CK14 at a time where the epithelium was pseudostratified and continuously expressed β-tubulin at the apical membrane (steps IV and V). Quantification of RT-PCR showed that the amount of α7 nAChR mRNA progressively increased during steps I to III ($P = 0.0183$) and then decreased during steps III to V ($P = 0.0388$; Friedman test) (Figure 3B).

Altogether, these observations indicate that the α7 nAChR expression is related to the differentiation of the airway epithelium and provide a rationale for investigating the implication of α7 nAChR in the differentiation of the airway epithelium.

α7 nAChR Expression/Activity Is Associated in Vivo *with the Remodeling of the Airway Epithelium*

The knockout mice model gives a good opportunity to investigate the effect of the α7 nAChR absence on the airway epithelium morphology and differentiation. In all tracheal sections from 1-year-old control and $\alpha7^{-/-}$ mice, three different phenotypes of the surface epithelium were observed. Phenotype 1 corresponds to a normal epithelium with two layers of nuclei and an epithelium height in the range of 25 to 35 μm. Phenotype 2 corresponds to a normal epithelium with three to four layers of nuclei and an epithelium height in the range of 40 to 55 μm. Phenotype 3 corresponds to a disorganized epithelium, with four to six layers of nuclei, an absence of the superficial columnar cell layer and an epithelium height in the range of 30 to 55 μm (Figure 4A). By using the *Griffonia simplicifolia* isolectin B4 (GSI-B4), which binds specifically to basal cells in the mouse tracheal epithelium,[30] we confirmed that phenotype 1 is characterized by one monolayer of basal cells, phenotype 2 contains one/two layers of basal cells, whereas GSI-B4 recognizes three/four layers of basal cells in phenotype 3 (Figure 4B). The percentage of phenotype 2 in the tracheal epithelium was decreased in $\alpha7^{-/-}$ mice as compared with control mice ($P = 0.0373$), whereas phenotype 3 was 3.8 times more expressed in $\alpha7^{-/-}$ mice ($P = 0.0174$) (Figure 4C), suggesting that the absence of the α7 nAChR in mice is associated with a more frequently observed hyperplasia of basal cells in the tracheal epithelium.

The allosteric model for nAChRs implies the equilibrium between channel resting, opening and desensitization.[31] Chronic exposure to nicotine results in both peaks of activation and long-term desensitization of nAChRs, but it is now believed that the overall effect of sustained smoking may result in nAChR desensitization and that chronic smoking maintains this desensitization, even if chronic nicotine exposure is also known to up-regulate nAChRs.[32] The α7 nAChR has been shown to be particularly susceptible to desensitization[33–35] and to up-regulation on nicotine exposure.[7,13,36] We thus postulated that in heavy smokers the potential nicotine-induced inactivation of the α7 nAChR may result in alterations in the airway epithelium differentiation. Indeed, in patients with chronic obstructive pulmonary disease (COPD) and with a significant smoking history, the airway epithelium is characterized by secretory cell or basal cell hyperplasia and squamous metaplasia (Figure 5). Hyperplasia of basal cells (area B and D) and squamous metaplasia (area C) are characterized by an overexpression of CK13 and CK14, desmoplakins 1 and 2, and by the absence of β-tubulin at the apex of the epithelium, confirming the absence of ciliated and polarized cells at the luminal surface. Whereas α7 nAChR expression is restricted to one basal cell layer at the margin of the pseudostratified epithelium (Figure 5 D, arrow), α7 nAChR is present up through most basal layers in areas of basal cell hyperplasia or squamous metaplasia (Figure 5, D and C, arrowheads). Similarly to what we observed during the *in vitro* epithelium differentiation (Figure 3 A), α7 nAChR expression in remodeled areas of the airway epithelium is mainly restricted to CK14-expressing basal cells.

These results suggest that the airway epithelium differentiation-associated α7 nAChR expression implicates the differentiation pathway of airway basal cells.

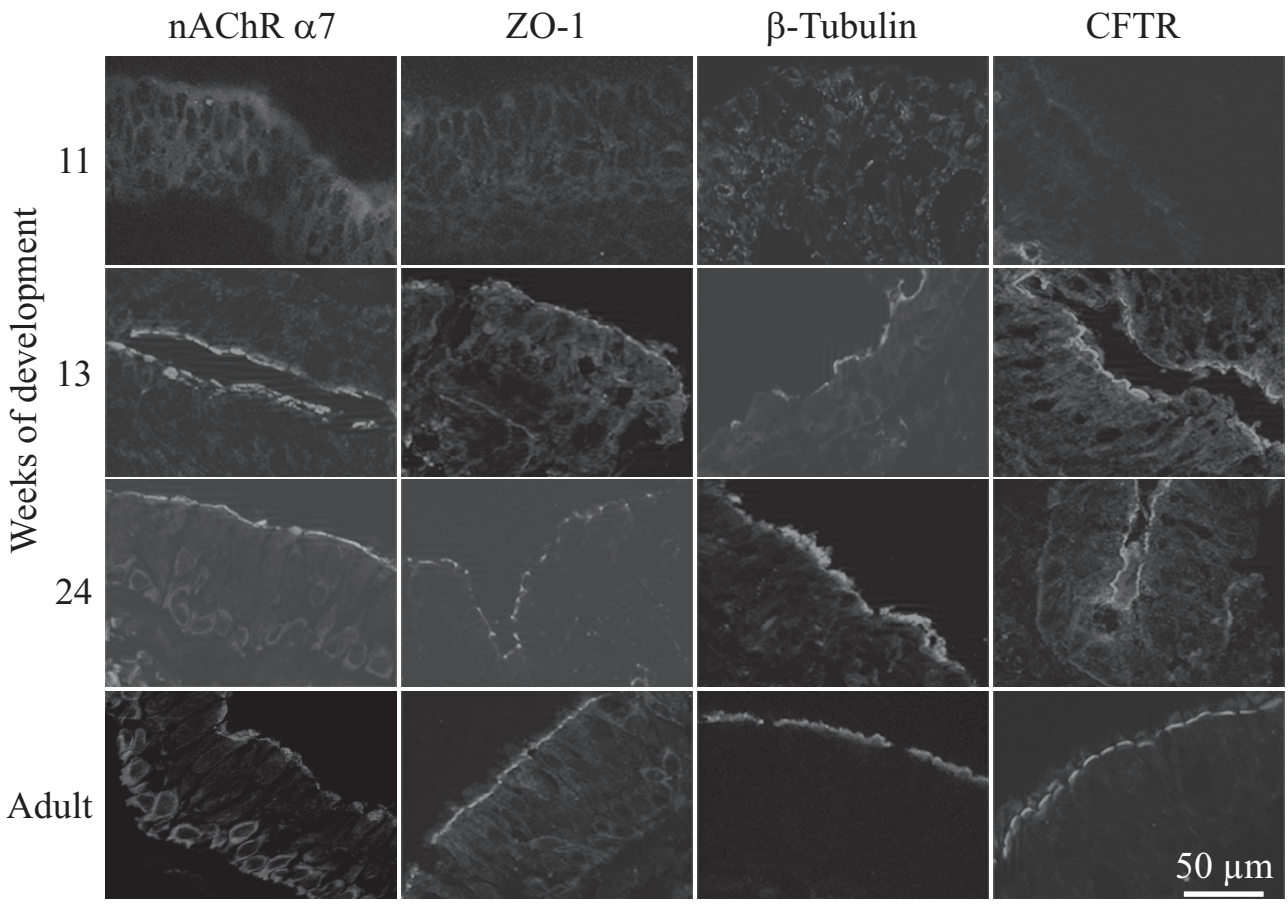

Figure 2. Expression of nAChR α7 during the fetal development of the human airway epithelium. nAChR α7, the protein ZO-1, β-tubulin, and CFTR were localized with Alexa 488 conjugates in human tracheal tissue samples at 11, 13, and 24 weeks of development and in samples from adult patients.

The α7 nAChR Is Involved in the Differentiation Pathway of the Airway Epithelium

To investigate the function of the α7 nAChR in the differentiation of the airway epithelium, we first studied the effect of αBTX, a toxin with a high affinity for the α7 nAChR, on human bronchial tissues undergoing repair. After inducing a local wound in fresh human bronchial tissues (*ex vivo* wound repair model), we observed that basal cells, characterized by the expression of CK13, are able to spread and migrate to progressively cover the wounded area. Similarly, we observed that the α7 nAChR is continuously expressed in basal cells in the unwounded pseudostratified epithelium at the lesion edge and in the repairing area (Figure 6A). Exposure of cultures to αBTX induced a progressive thickening of the epithelium in the repairing areas, with two to three and then four to six cell layers after 3 and 4 days, respectively, as compared with one to three cell layers observed in the control cultures. In both control- and αBTX-exposed cultures, all repairing cells contained CK13, but more cells expressed Ki-67, a proliferation marker, in their nuclei when exposed to αBTX, especially after 4 days in culture (Figure 6B). These observations suggest that the pharmacological inactivation of α7 nAChR up-regulates basal cell proliferation and modulates the early events of epithelium regeneration. To analyze in more details and to quantify these effects, we used an *in vitro* air-liquid interface culture model of airway epithelium regeneration.

When analyzing the effect of the chronic exposure to αBTX on the regeneration of the human airway epithelium (*in vitro* air-liquid interface culture model), we first observed that αBTX induced a dramatic increase in cell proliferation in the early phases of epithelial regeneration. Indeed, in the presence of 5 μmol/L αBTX, we observed a significant increase of the cell number ($P < 0.05$) at day 5 (+626%), day 7 (+206%), day 9 (+123%), day 11 (+23%), day 16 (+11%), and day 23 (+10%) (Figure 7A). After 2 days in culture, we identified more Ki-67-positive nuclei in αBTX-exposed cells than in control cells (Figure 7B), suggesting that αBTX was likely to favor cell cycle progression rather than inhibiting apoptosis. Moreover, we observed that all cells expressing Ki-67 in the nucleus, were those expressing CK13, a marker of airway basal cells (Figure 7B), suggesting that αBTX induced basal cell proliferation during the initial step of epithelial regeneration. Figure 7C (control) depicts the evolution of the epithelium morphology during the regeneration process in the absence of αBTX. On day 16, the control cultures exhibit three to four layers of cuboidal undifferentiated cells. On day 23, columnar cells and some ciliated cells (arrowheads) are observed in the upper part of the epithelium. Finally on day 33, the epithelium is pseudostratified, ciliated cells are present all along the

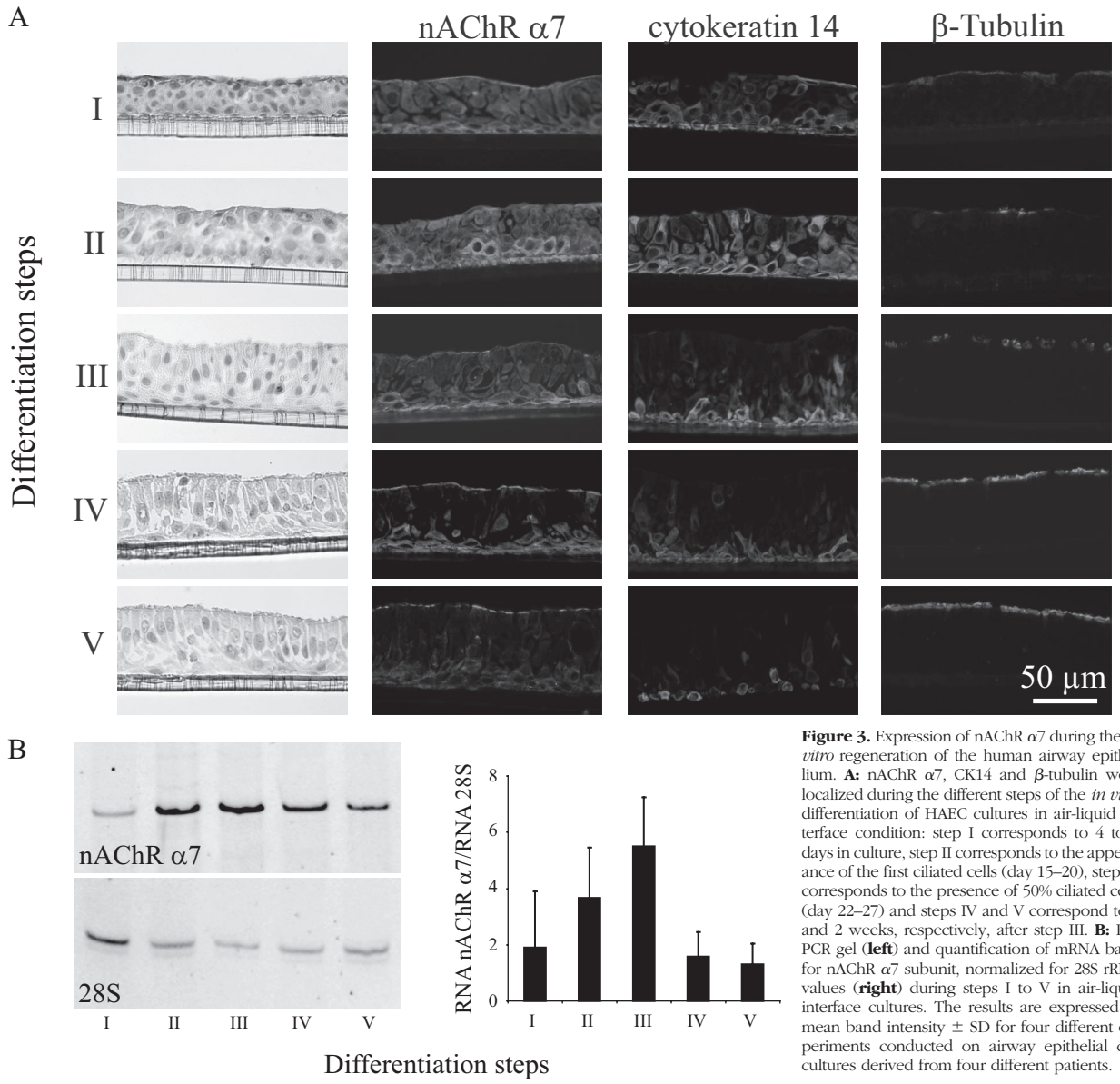

Figure 3. Expression of nAChR α7 during the *in vitro* regeneration of the human airway epithelium. **A:** nAChR α7, CK14 and β-tubulin were localized during the different steps of the *in vitro* differentiation of HAEC cultures in air-liquid interface condition: step I corresponds to 4 to 7 days in culture, step II corresponds to the appearance of the first ciliated cells (day 15–20), step III corresponds to the presence of 50% ciliated cells (day 22–27) and steps IV and V correspond to 1 and 2 weeks, respectively, after step III. **B:** RT-PCR gel (**left**) and quantification of mRNA band for nAChR α7 subunit, normalized for 28S rRNA values (**right**) during steps I to V in air-liquid interface cultures. The results are expressed as mean band intensity ± SD for four different experiments conducted on airway epithelial cell cultures derived from four different patients.

epithelium, CK13 and CK14 and desmoplakins 1 and 2 are essentially expressed in the basal layer of basal cells while β-tubulin and α subunit of the epithelial sodium channel (α-ENaC) are expressed all along the apical membrane of columnar cells, suggesting that the regenerated epithelium is now fully differentiated (Figure 7D, control). When epithelial regeneration was performed in the presence of αBTX, added from day 1, the epithelium was pluristratified and composed of five to six and seven to eight layers of cuboidal cells at days 16 and 23, respectively, with some very flat cells observed at the top of the epithelium on day 23. After 33 days of αBTX exposure, the epithelium still consisted in 8 to 10 layers of cuboidal and polyhedral cells, most of them overexpressing CK13 and CK14 and desmoplakins 1 and 2 in the basal layers whereas the apical expression of β-tubulin and α-ENaC was greatly reduced (Figure 7, C and D, αBTX d1), a situation similar to that observed *in vivo* in squamous metaplasia of the airway epithelium from smoking patients with COPD (Figure 5C). Moreover, p63, present in airway basal cells and whose expression is decreased during squamous cell differentiation,[37] was expressed in the control basal cells but absent in αBTX-exposed cultures. Transglutaminase-1, a well-known marker of squamous differentiation of the respiratory tract,[38] is expressed at low level in the control cultures, but overexpressed in the presence of αBTX (Figure 7D). The forkhead transcription factor Foxj1 is required for ciliogenesis and also involved in the organization of the apical membrane.[39] In the differentiated pseudostratified epithelium, Foxj1 is present and expressed at the same level in most cell nuclei of the epithelium. In αBTX-exposed cultures, Foxj1 is still present in the basal progenitor cells of the epithelium, but its expression is much lower in the intermediate cells and finally absent in the upper part of the epithelium (Figure 7D). In summary,

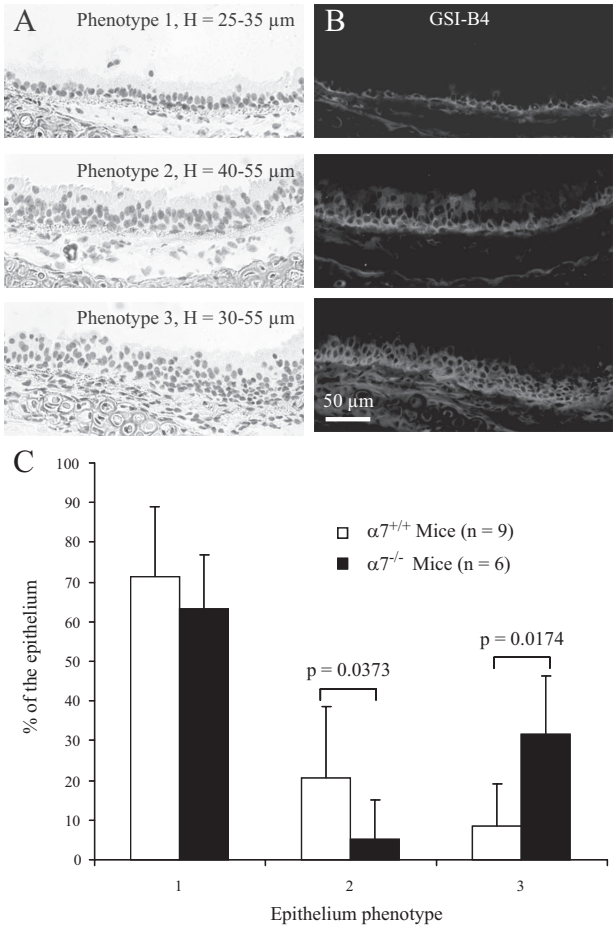

Figure 4. Morphological characterization of the tracheal epithelium in $\alpha7^{+/+}$ and $\alpha7^{-/-}$ mice. **A:** Histological hematoxylin-stained sections of the upper trachea illustrating the three different epithelial phenotypes observed in 1-year-old mice. **B:** Binding of the FITC-conjugated GSI-B4 lectin to cryosections of mouse trachea. Each panel is matched with the corresponding hematoxylin-stained sections presented at the same level in **A**. **C:** Quantification (mean ± SD) of each phenotype 1, 2, and 3, expressed as percentage of the total length of the tracheal epithelium in cross-sections of trachea from control $\alpha7^{+/+}$ mice (white bars, $n = 9$) and from $\alpha7^{-/-}$ mice (black bars, $n = 6$). The significance of the differences between control $\alpha7^{+/+}$ mice and $\alpha7^{-/-}$ mice was determined using the Mann-Whitney test.

adding αBTX from day 1-stimulated cell proliferation during the initial steps of the regeneration and prevented the differentiation of the normal pseudostratified airway epithelium. To investigate in more details the relationship between the initial stimulation of cell proliferation and the subsequent alteration of epithelium differentiation, we tested the effect of adding chronically αBTX from day 11, ie, after ~50% of cell proliferation has already occurred in the control culture (Figure 7A). When adding αBTX from day 11, we did not observed any subsequent modification of cell proliferation and the evolution of epithelium morphology and the distribution of differentiation markers were similar to those observed in the control culture, even after a 22-day exposure to αBTX (Figure 7 A, C, and D, αBTX d11), suggesting that cell proliferation needs to be altered by αBTX, during the first steps of the epithelium regeneration, to induce an alteration in epithelium differentiation.

Finally, we investigated the effect of α7 nAChR absence on the regeneration of the tracheal epithelium in $\alpha7^{+/+}$ and $\alpha7^{-/-}$ mice. Desquamation of the mouse tracheal epithelium was induced after instillation of a polydocanol solution.[25] Figure 8A depicts the morphology of regenerating tracheal epithelium in $\alpha7^{+/+}$ and $\alpha7^{-/-}$ mice from days 0 to 8 after polydocanol instillation. Before injury, both $\alpha7^{+/+}$ and $\alpha7^{-/-}$ 8-week-old mice presented the same pseudostratified morphology of the tracheal epithelium with one to two layers of nuclei. On day 2, only flat cells spreading over the denuded basement membrane are observed. On day 4, 3 to 4 and 8 to 10 cell layers are present in the control $\alpha7^{+/+}$ mice and in $\alpha7^{-/-}$ mice, respectively, suggesting that an active cell proliferation has occurred with a more significant effect observed in $\alpha7^{-/-}$ mice. The first columnar cells are observed in $\alpha7^{+/+}$ mice, whereas the epithelium in $\alpha7^{-/-}$ mice is still undifferentiated. On day 6, the first basal cells are observed only in $\alpha7^{+/+}$ mice. On day 7, the epithelium in $\alpha7^{+/+}$ mice is almost fully differentiated whereas that of $\alpha7^{-/-}$ mice begins to pseudostratify with the appearance of the first basal cells. On day 8, the epithelium in $\alpha7^{+/+}$ mice and in $\alpha7^{-/-}$ mice has a similar pseudostratified structure. We measured the epithelium height during the whole process of regeneration (Figure 8B). On day 4, the epithelium is thicker both in $\alpha7^{+/+}$ and $\alpha7^{-/-}$ mice ($P = 0.0339$) as compared with day 0. Then, we observed a progressive and significant decrease of the epithelium height between day 4 and day 8 both in $\alpha7^{+/+}$ mice ($P = 0.0315$) and in $\alpha7^{-/-}$ mice ($P = 0.0067$), with a thicker epithelium in $\alpha7^{-/-}$ mice on day 4 and day 6 ($P = 0.0209$). Finally on day 8, the epithelium height in $\alpha7^{-/-}$ mice (33.9 ± 3.4 μm) is still higher as compared with day 0 (27.5 ± 1.3; $P = 0.0339$), whereas the epithelium height in $\alpha7^{+/+}$ mice (30.0 ± 4 μm) is similar to that observed on day 0 (28.8 ± 0.8; $P > 0.99$). Immunostaining for caveolin-1, a marker of basal cells in the mouse tracheal epithelium,[40] confirmed a basal cell hyperplasia more pronounced in $\alpha7^{-/-}$ mice than in $\alpha7^{+/+}$ mice both on days 4 and 6, whereas a single basal cell layer is observed on day 8 (Figure 8C). These results suggest that the regeneration of the airway epithelium in $\alpha7^{-/-}$ mice is altered and characterized by a transient hyperplasia of basal cells.

α7 nAChR Regulates the Proliferation of Airway Epithelial Basal Cells

We have observed that α7 nAChR regulates the proliferation of HAECs in air-liquid interface culture and that the proliferating cells are mainly composed of basal epithelial cells (Figure 7, A and B). Because $\alpha3\alpha5\beta2$ nAChR is also expressed in basal epithelial cells and involved in the wound repair process of the injured airway epithelium,[19] we investigated the respective and potential role of α7 nAChR and $\alpha3\alpha5\beta2$ nAChR in basal cell proliferation by studying the effect of different nAChR antagonists, αBTX[10,26,27] and methyllycaconitine[41] for the α7 nAChR, α-connotoxin MII for the $\alpha3\beta2$ nAChR,[16] and mecamylamine for all $\alpha_x\beta_y$ heteropentameric nAChRs,[42] on the *in vitro*

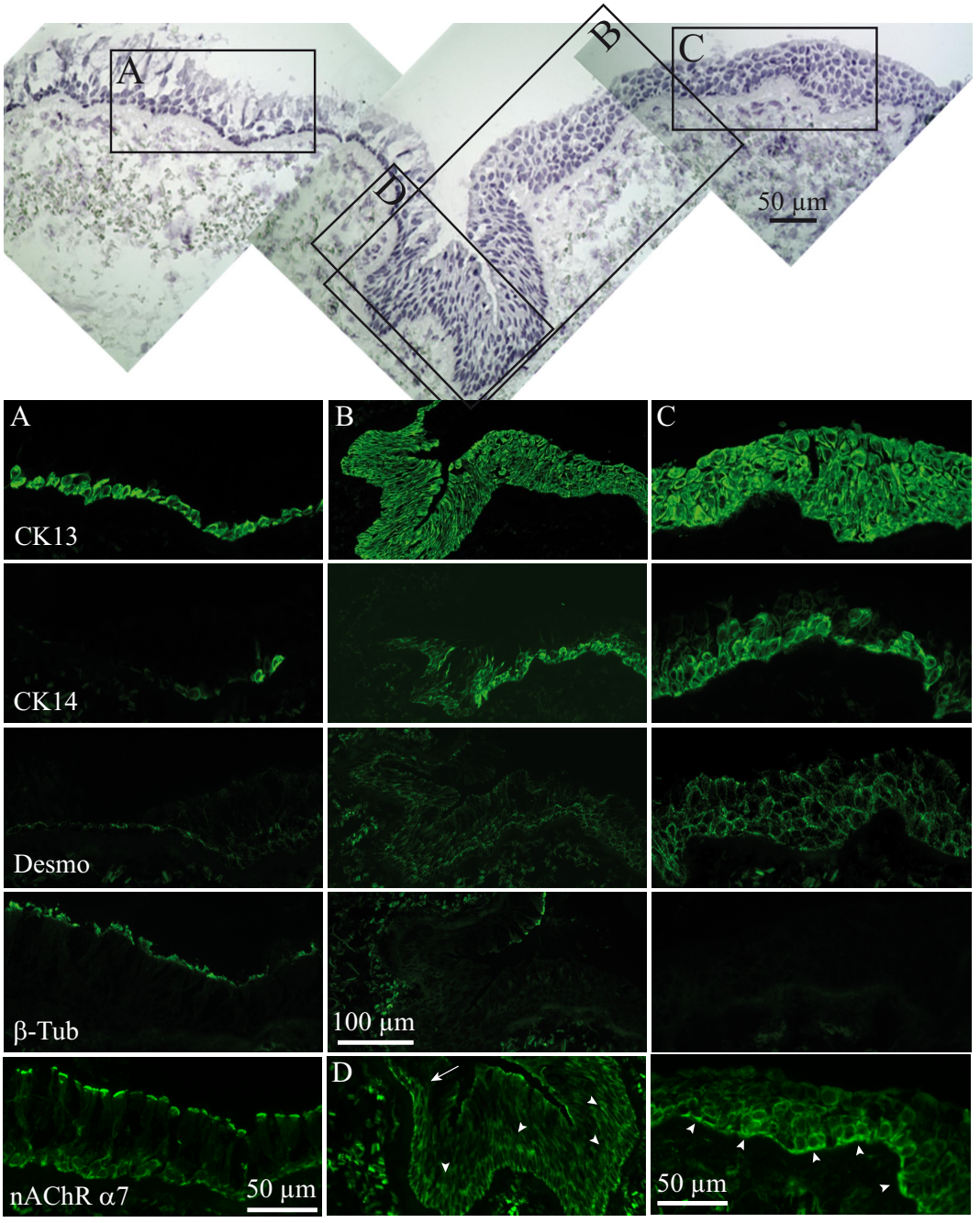

Figure 5. nAChR α7 in a smoker-derived remodeled human airway epithelium. The localization of nAChR α7, CK13 and CK14, desmoplakins 1 and 2 (Desmo), and β-tubulin was studied by immunofluorescence with Alexa 488 conjugates (**green color** in **dark panels**) in a human bronchial tissue sample derived from a patient with a significant smoking history and presenting different types of remodeling: normal pseudostatified epithelium with hyperplasia of secretory cells (**A**), hyperplasia of basal cells and squamous metaplasia (**B**), and squamous metaplasia (**C**). **D** reports α7 nAChR distribution in the area of basal cell hyperplasia. α7 nAChR is expressed in the basal cell layer in the pseudostratified epithelium (**D**, **arrow**) and present in most basal layers in areas of basal cell hyperplasia or squamous metaplasia (**D** and **C**, **arrowheads**).

proliferation of isolated HAECs (Figure 9A) and of sorted human airway basal epithelial cells (Figure 9B). We did not observe any significant effect of both α-connotoxin MII and mecamylamine on cell proliferation when tested from 0.1 to 10 μmol/L. On the contrary, αBTX exposure dose-dependently increased cell proliferation both in isolated HAECs (Figure 9A) and in sorted human airway basal epithelial cells (Figure 9B), with a maximal effect observed for a 10 μmol/L concentration. Both αBTX and methyllycaconitine, two competitive antagonists of α7 nAChRs, stimulated basal cell proliferation (Figure 9B). Similarly, exposure of 16HBE14o-cells, a normal human airway cell line[43] expressing the basal airway cell markers CK13 and CK14 (Figure 10 A), to 0.1–10 μmol/L αBTX significantly increased ($P = 0.0074$, Friedman test) cell proliferation in a dose-dependent way (Figure 10B). Transfection of the 16HBE14o-cell line with two different small interfering RNAs (siRNAs), Si1 and Si2, targeted to

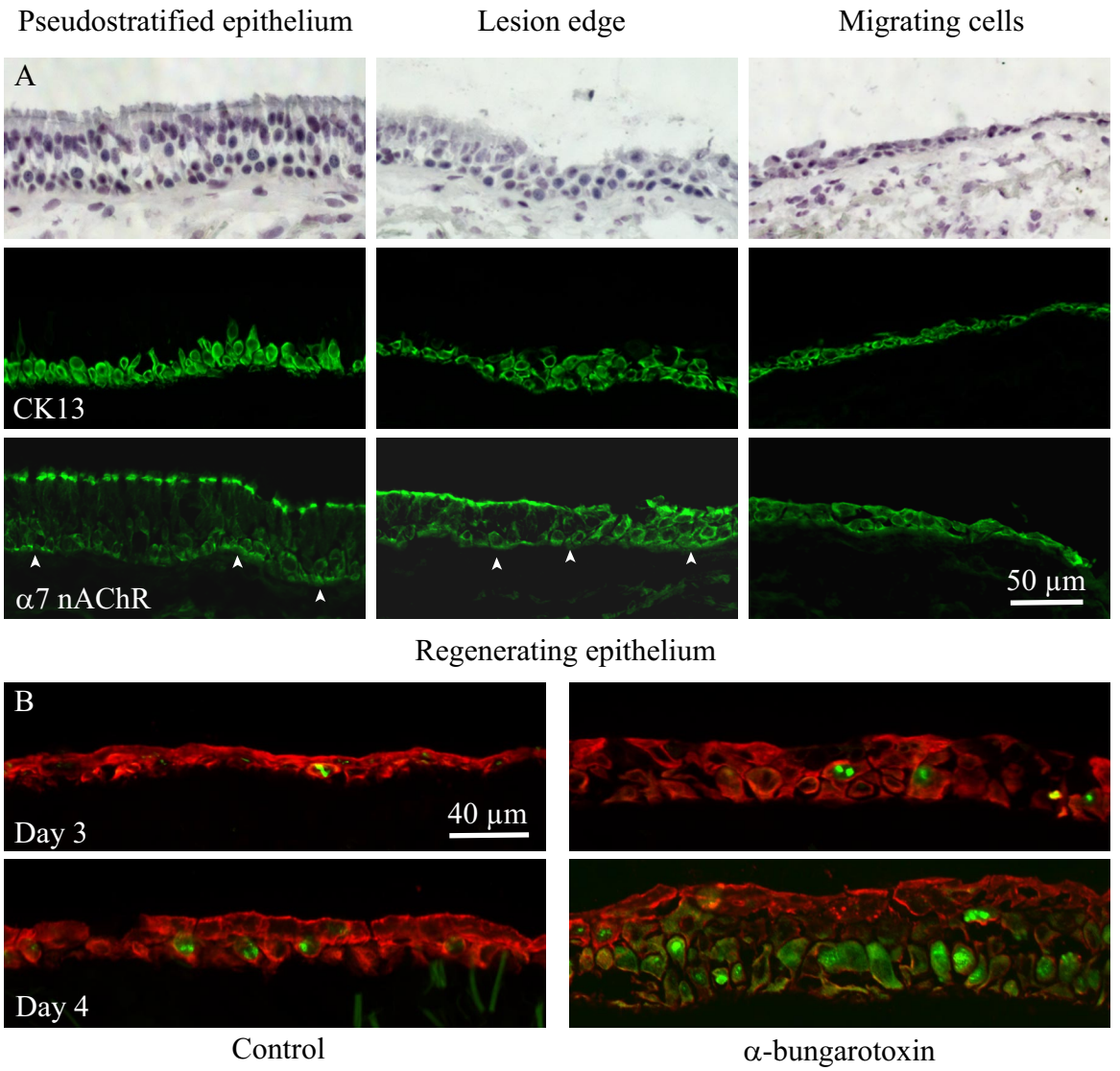

Figure 6. Effect of αBTX on the *ex vivo* wound repair of the human respiratory epithelium. Normal human bronchial tissue samples were locally wounded (*ex vivo* wound repair model) and maintained in culture. **A:** After 2 days in culture, the localization of CK13 and nAChR α7 was studied by immunofluorescence with Alexa 488 conjugates (**green color** in **dark panels**) in stationary cells in the unwounded pseudostratified normal epithelium (**left column**), in the transition area between the normal unwounded epithelium and the migrating cells (**middle column**) and in migrating cells in the wounded area (**right column**). **Arrowheads** denote expression of nAChR α7 in basal cells present in the unwounded pseudostratified epithelium and at the lesion edge. **B:** After 3 and 4 days in culture in the presence (**right panels**) or absence (control, **left panels**) of 5 μmol/L αBTX, the localization of CK13 (Alexa 594, **red color**) and of the Ki-67 protein (Alexa 488, **green color**) was studied by immunofluorescence in the regenerating epithelium, ie, in cells located in the initially wounded area of the injured epithelium.

mRNA encoding the α7 nAChR subunit, significantly inhibited protein level for α7 nAChR by 56 and 41%, respectively (Figure 10C) and stimulated cell proliferation over a 1- to 6-day period (Figure 10D), as compared with scramble siRNA controls. On the contrary, transfection of the 16HBE14o- cell line with the α7 nAChR cDNA significantly increased by 157% α7 nAChR protein level (Figure 10E) and decreased cell proliferation by 49 and 25%, 3 and 6 days after transfection, respectively, as compared with transfection with the pCMV6 empty vector (Figure 10F).

Discussion

The remodeling of the airway epithelium, described as abnormal plasticity, includes basal cell and goblet cell hyperplasia, and squamous cell metaplasia.[44] These changes involve that any delay in the repair of the injured epithelium, or disruption in proliferation and differentiation, may affect the dynamic process of regeneration. In this study, we demonstrate that the α7 nAChR, expressed in human airway basal cells, is essential for the regeneration of a normal pseudostratified epithelium by regulating basal cells proliferation *in vivo* and *in vitro*. Our findings also suggest that alteration of α7 nAChR expression or activity in airway basal cells is involved in the airway epithelium remodeling, namely basal cell hyperplasia and squamous metaplasia.

In animal models, it has been shown that airway basal cells have the potential to proliferate and to differentiate into most cell types of the mucociliary epithelium to re-

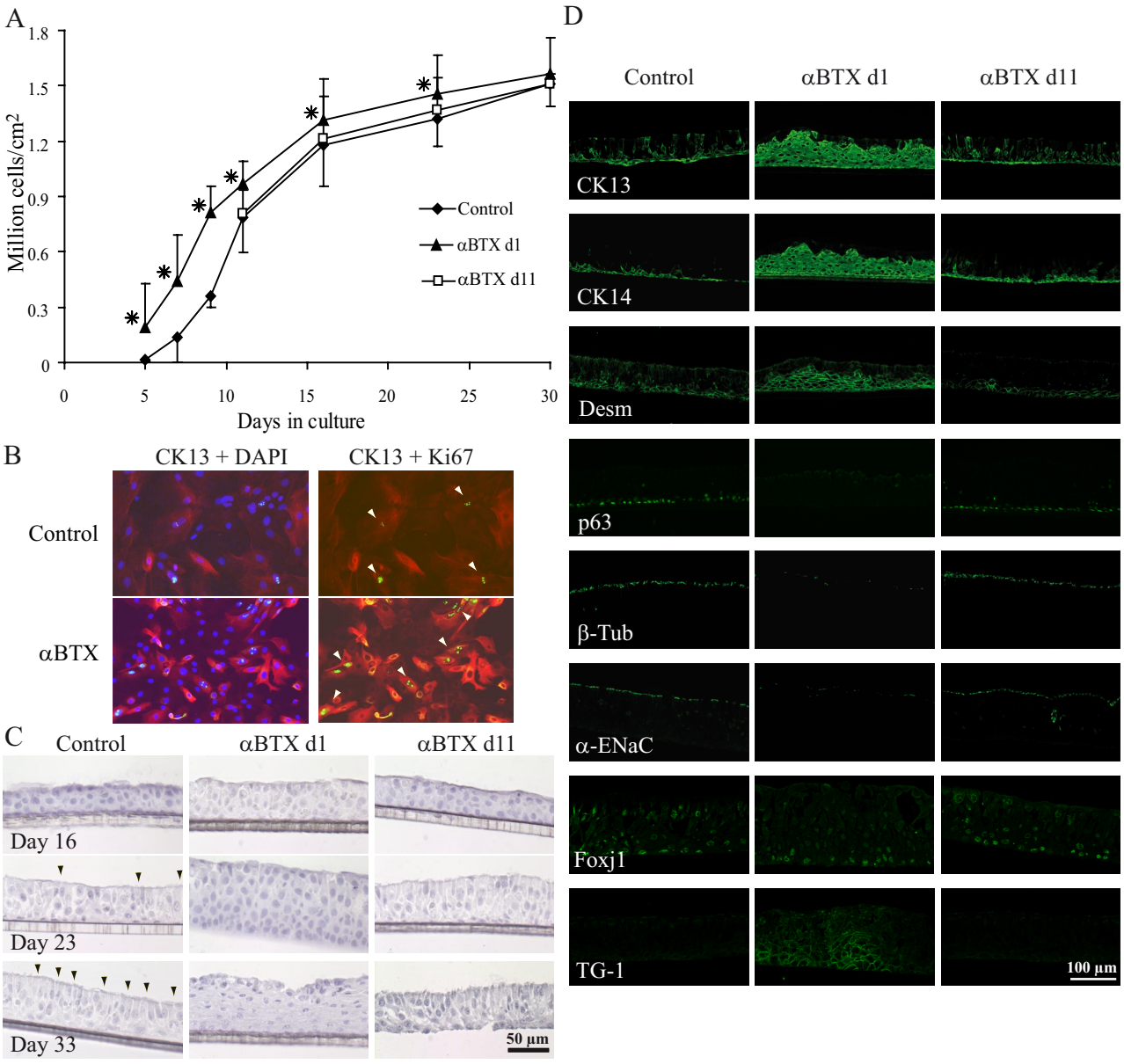

Figure 7. Effect of αBTX on the *in vitro* regeneration of the human respiratory epithelium. HAECs were isolated and cultured in air-liquid interface in the absence (control) or in the presence of 5 μmol/L αBTX added from day 1 (αBTX d1) or from day 11 (αBTX d11). **A:** Cells in each condition were counted over a 30-day period in culture. The results are expressed as mean cell number ± SD for five different experiments conducted on primary cultures of HRECs derived from five different patients. The significance of the differences between αBTX-exposed cultures and control cultures was determined using Wilcoxon rank test ($^{*}P < 0.05$). **B:** CK13 (**red color:** Alexa 594 conjugate), Ki-67 (**green color:** Alexa 488 conjugate), and cell nuclei (**blue color:** 4′,6′-diamino-2-phenylindole) immunostaining of HRECs after 2 days in control and αBTX-exposed cultures; **arrowheads** point to Ki-67 immunoreactivity in CK13-positive cells. **C:** Morphology of the regenerated epithelium after 16, 23, and 33 days in culture in the different conditions (control, αBTX d1 and αBTX d11). **Arrowheads** point to ciliated cells. **D:** CK13, CK14, desmoplakins 1 and 2 (Desm), P63, β-tubulin (β-Tub), α-ENaC, Foxj1, and transglutaminase-1 (TG-1) immunostaining with Alexa 488 conjugates of HRECs after 33 days in culture in air-liquid interface condition.

generate its structure.[45–48] More recently, *in vivo* and *in vitro* studies demonstrated that human adult airway basal cells, as opposed to ciliated and secretory cells, are able to proliferate and to reconstitute a fully differentiated and functional mucociliary airway epithelium and may be considered as progenitors of the human airway epithelium.[2] We have observed that airway epithelial cells, migrating to repair a wound (*ex vivo* wound repair model) or adhering and proliferating to further regenerate the epithelium (air-liquid interface culture model), present basal cell characteristics and continuously express α7 nAChR. Although α7 nAChR has been shown to be expressed at the apex of the rat tracheal epithelium[49] or of nicotine-treated monkey airway epithelium,[13] the expression of α7 nAChR in airway basal cells has never been reported before. Although α7 nAChR is expressed in migrating basal cells repairing a wound, we previously observed that α7 nAChR is not involved in the first step of epithelium regeneration, ie, cell spreading and migration, which implies the α3α5β2 nAChR.[19] Here, we show that α7 nAChR expressed in airway basal cells is involved in the epithelium differentiation.

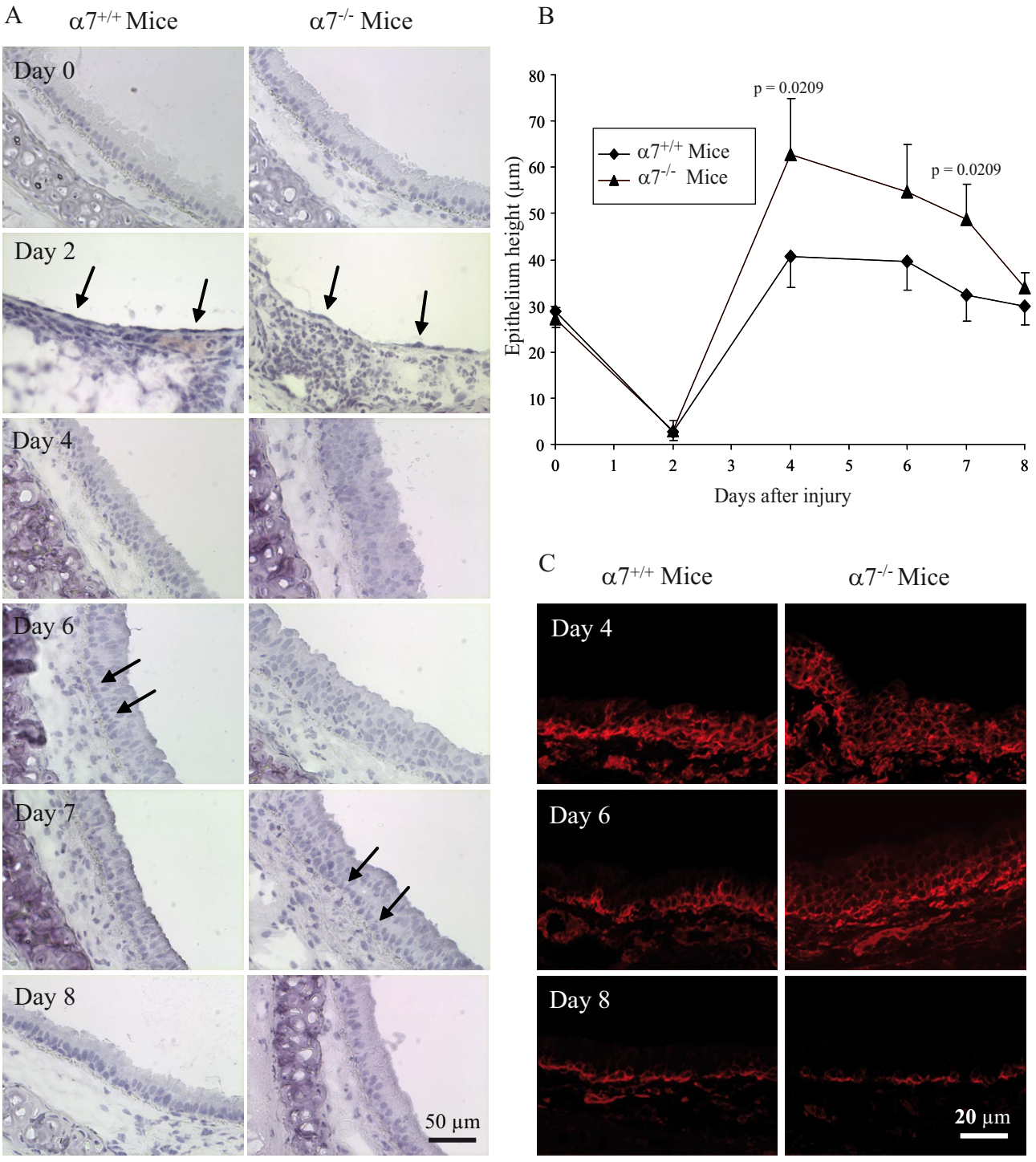

Figure 8. Regeneration of the tracheal epithelium after exposure to polydocanol in $\alpha7^{+/+}$ and $\alpha7^{-/-}$ mice. **A:** Histological hematoxylin-stained sections of upper trachea from day 0 (just before polydocanol treatment) to day 8 in 8-week-old mice. **Arrows** in day 2 denote only flat cells spreading over the denuded basement membrane are observed both in $\alpha7^{+/+}$ mice and in $\alpha7^{-/-}$ mice. **Arrows** in day 6 denote the first basal cells observed in $\alpha7^{+/+}$ mice. **Arrows** in day 7 denote the appearance of the first basal cells in $\alpha7^{-/-}$ mice. **B:** Variation of epithelium height during the epithelium regeneration. Results are expressed as mean epithelium height ± SD for four to five mice for each time. The significance of the differences between control $\alpha7^{+/+}$ mice and $\alpha7^{-/-}$ mice was determined at each time using the Mann-Whitney test. **C:** Identification of caveolin-1 in cryosections by immunofluorescence with an Alexa 594 conjugate.

The expression of $\alpha7$ nAChR is definitely associated with the differentiation state of the airway epithelium. The highest detectable $\alpha7$ nAChR expression, both during the human airway fetal development and during the *in vitro* regeneration of the human pseudostratified epithelium, is observed when airway epithelial differentiation takes place. Otherwise, $\alpha7^{-/-}$ mice are viable and the airway epithelium morphology is similar in $\alpha7^{-/-}$ mice and in control mice, at least during the first weeks of life, suggesting that $\alpha7$ nAChR expression is not essential for

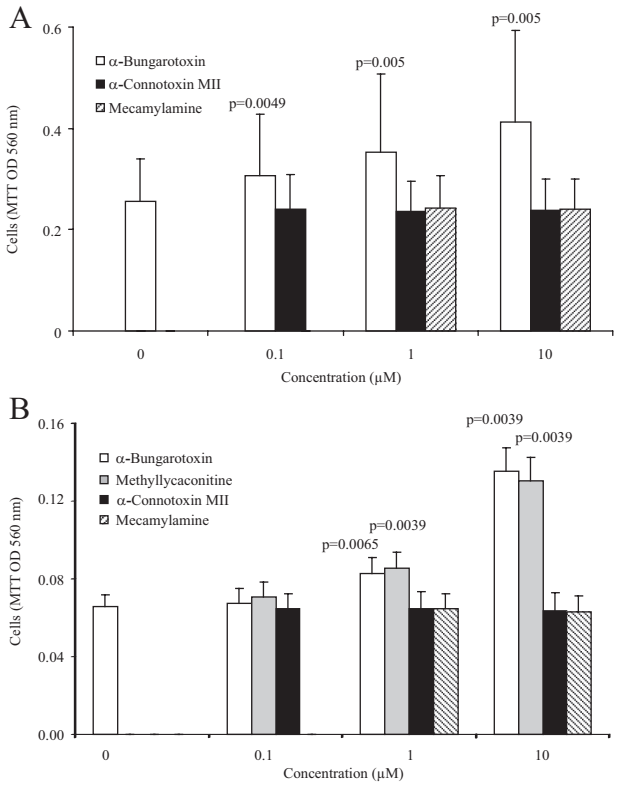

Figure 9. Effect of nicotinic antagonists on the *in vitro* proliferation of HAECs. The proliferation of HAECs, isolated from normal human bronchial tissues (**A**) or of basal cells, sorted by FACS (tetraspanin CD151$^+$ and tissue factor$^+$) from a normal human bronchial tissue sample (**B**) was studied with the 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) technique on multiwell plates in the presence of increasing concentrations of αBTX, methyllycaconitine, α-connotoxin MII, and mecamylamine (**A** and **B**). The results are expressed as mean OD at 560 nm ± SD for 10 different experiments conducted on primary cultures of HRECs derived from 10 different patients (**A**) or for one experiment with sorted basal epithelial cells, run in sextuplicates (**B**). The significance of the differences between control and antagonist exposed-cells was determined using the Wilcoxon rank test (**A**) and the Mann-Whitney test (**B**).

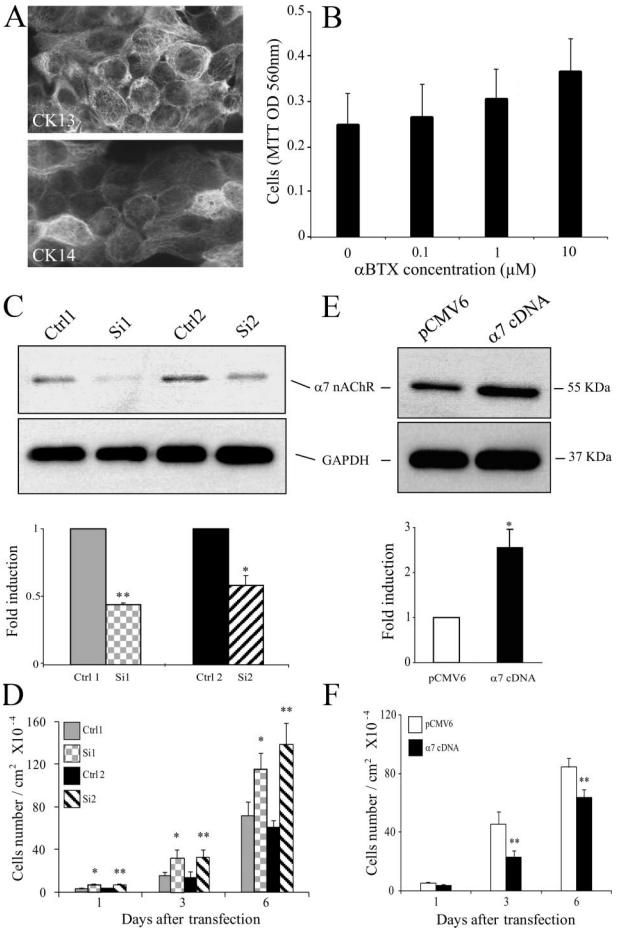

Figure 10. Effect of αBTX, of siRNA, and of cDNA specific for nAChR α7 on the proliferation of the normal airway cell line 16HBE14o-. **A:** CK13 and CK14 immunostaining of 16HBE14o-cells in culture. **B:** 16HBE14o-cells were cultured for 6 days in multiwell plates in the presence of increasing concentrations of αBTX, and the cell number was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) method. Results are expressed as mean OD at 560 nm ± SD for four different αBTX-treated cultures. **C–F**: 16HBE14o-cells were transiently transfected either with two different siRNA sequences against nAChR α7 subunit (Si 1 and Si 2), or with two scrambled control siRNAs (Ctrl 1 and Ctrl 2) (**C** and **D**), or with the α7 cDNA expression vector, or the empty control vector (pCMV6) (**E** and **F**). **C** and **E:** Immunoblots (**top panels**) and quantification of nAChR α7 subunit, normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) values (**bottom panels**) 6 days after transfection. Data are expressed as fold induction for α7 (Si1, Si2, or cDNA) transfectants relative to the control (Ctrl1, Ctrl2, or pCMV6) transfectants. The significance of the differences between transfected cells and the corresponding controls was determined using the one simple *t*-test ($^*P < 0.05$; $^{**}P < 0.01$). **D** and **F:** Transfected cells were counted on days 1, 3, and 6 after transfection. The results are expressed as mean cell number ± SD for four different transfected cultures. The significance of the differences between α7 siRNA or α7 cDNA-transfected cells and their corresponding controls was determined using the Mann-Whitney test ($^*P = 0.0339$; $^{**}P = 0.0209$).

normal airway development or that the missing function of α7 nAChR in α7$^{-/-}$ mice is compensated by up-regulated expression of other nAChR subunits as described in keratinocytes.[50] Nevertheless, chronic prenatal nicotine exposure increases pulmonary α7 nAChR expression and alters fetal lung development in monkeys.[13] Chronic exposure to submicromolar concentration of nicotine is also known to irreversibly inactivate many nAChRs, including α7 nAChR,[35] and inactivation of α7 nAChR alters pulmonary morphogenesis.[15] Similarly, we have observed that the chronic inactivation of α7 nAChR by αBTX prevents the *in vitro* differentiation of the normal human pseudostratified epithelium and that the absence of α7 nAChR in mice induces modifications of the epithelium phenotype and an altered epithelial regeneration after wounding. We can therefore infer that α7 nAChR plays a potential role in the airway epithelium differentiation. Surprisingly, in all these *in vivo* and *in vitro* approaches, we observed that the absence or the inactivation of α7 nAChR is characterized by either a transient or an established hyperplasia of basal epithelial cells, which led us to investigate the potential role of the α7 nAChR in the regulation of airway epithelial basal cell proliferation.

α7 nAChR appears to be a key negative regulator of airway epithelial basal cell proliferation: 1) inactivating α7 nAChR *in vitro* with αBTX stimulates cell proliferation in the early phases of the epithelial regeneration and the corresponding proliferating cells have phenotypic characteristics of basal epithelial cells, 2) regeneration of the airway epithelium in mice lacking α7 nAChR is characterized by a transient hyperplasia of basal cells, 3) airway epithelium in aged α7$^{-/-}$ mice more frequently presents areas of basal cell hyperplasia, and 4) *in vitro*, the prolif-

eration of sorted human airway basal cells is stimulated by αBTX and methyllycaconitine but not by α-Conotoxin MII nor mecamylamine, suggesting that only α7 nAChR regulates the proliferation of basal cells in the normal human airway epithelium. Similarly, inhibition of the α7 nAChR pathway favored cell cycle progression in epidermal keratinocytes.[50] On the contrary, many authors have noticed that α7 is the main nAChR subunit that mediates the proliferative effects of nicotine in cancer cell lines.[12] This α7 nAChR-mediated stimulatory effect of nicotine was also observed in commercialized normal bronchial epithelial cells, after these cells have been amplified after isolation.[51] At the opposite, we always observed a stimulatory proliferative action of αBTX on primary cultures of freshly isolated HAECs and on purified human airway basal cells. We also observed the same proliferation stimulatory effect of both αBTX and α7 nAChR-specific siRNAs on the 16HBE14o-cell line, a normal human airway cell line expressing the basal airway cell markers CK13 and CK14, whereas transfection with the α7 nAChR cDNA inhibited cell proliferation. We observed a stimulatory proliferative effect of nicotine both on HAECs and isolated human airway basal cells only when cells were incubated for 7 to 15 days with high nicotine concentrations (10 to 100 μmol/L), conditions which may have favored α7 nAChR inactivation (data not shown). This antagonist-like effect of nicotine, on over exposure of HAECs to nicotine, has been already described by Zia et al.[7] These results suggest that the regulation of cell proliferation by α7 nAChR may be quite different in the normal airway epithelium and in primary cultures of normal HAECs, on the one hand, and in lung cancer and cell lines derived from lung cancers, on the other hand.

Our results raise the concept of coupling between cell growth and differentiation. In the normal pseudostratified tracheobronchial epithelium, a strict balance exists between cellular proliferation and differentiation.[44,52] This balance is likely maintained via equilibrium between positive and negative growth regulatory factors and factors controlling differentiation.[53] It is generally accepted that an inverse relationship exists between growth and differentiation in the airway epithelium.[52,54] For example, after mechanical or toxic injury, the balance between proliferation and differentiation is disturbed, and the epithelium undergoes hyperplasia followed by transient squamous metaplasia,[55–60] a situation similar to our observation after αBTX exposure: that is, after an increased cell proliferation, the regenerated airway epithelium ends up with squamous metaplasia. Interestingly, we have observed that, although the airway epithelium undergoes squamous metaplasia *in vitro*, Foxj1, a transcription factor involved in ciliated cells differentiation,[61,62] is down-regulated. Regulation of Foxj1 expression may thus be a key element in the αBTX-induced epithelium squamous metaplasia. We have observed that αBTX-induced squamous metaplasia takes place only when adding the α7 nAChR antagonist during the proliferation step of the airway epithelium regeneration. It has been proposed that the induction of squamous metaplasia after injury of the tracheobronchial epithelium involves enhanced proliferation of a $p63^+$/$CK14^+$/$CK5^+$/BS-I-$B4^+$ subpopulation of basal cells.[37] Indeed, we have observed that α7 nAChR blockade induces expression of squamous metaplasia phenotype in cultured HAECs, which was preceded by $CK14^+$ basal cell hyperplasia. Interestingly, constitutive expression of the human CK14 gene in the mouse airway epithelium induced tracheal basal cell hyperplasia and squamous metaplasia.[63] Of note, the lack of p63 expression in αBTX-induced squamous metaplasia is in agreement with the hypothesis that down-regulation of p63 might be necessary for basal cells to differentiate into squamous cells.[37] Among the many factors that have been identified and regulate positively or negatively the growth and differentiation of airway epithelial cells by autocrine or paracrine mechanisms, transforming growth factor β (TGF-β), a major cytokine involved in driving squamous metaplasia, is of particular interest. Jetten et al.[38] and Masui et al.[64] have indeed shown that TGF-β exposure or overexpression in airway epithelial cells induces terminal cell division and favors the expression of a squamous phenotype. Similarly in the present study, αBTX-induced hyperproliferation is followed by a period in which cells stop proliferating and undergo squamous differentiation, suggesting that these changes in the differentiation pathway, when α7 nAChR is absent or inactivated, may be related to TGF-β overexpression. In addition, we have observed that the α7 nAChR blockade enhances the expression of transglutaminase, a marker of squamous differentiation known to be up-regulated by TGF-β.[38] Moreover, up-regulation of TGF-β by nicotine is mediated by α7 nAChR during atrial remodeling,[65] TGF-β up-regulates CK14 expression and promotes the expression of basal cell phenotype[66] and inhibits p63 function.[67] Taken together, our results suggest that α7 nAChRs inactivation can initiate an alternative program of the basal cell differentiation process leading to squamous differentiation and that this process may be associated to down-regulation of Foxj1 and/or up-regulation of TGF-β.

Our data suggest that α7 nAChR is critical for the renewal of the normal respiratory epithelium by repressing basal cell proliferation and stimulating their differentiation. After an acute injury, the regeneration of the airway epithelium in $\alpha7^{-/-}$ mice is delayed, transiently abnormal, and characterized by a basal cell hyperplasia. On the long term and after many epithelium renewals, absence or inactivation of the α7 nAChR may progressively induce the frequent remodeling of the airway epithelium observed in old $\alpha7^{-/-}$ mice: hyperplasia of basal cells and squamous metaplasia. α7 nAChR inactivation, during the *in vitro* regeneration of the human airway epithelium, also induces dramatic morphological changes leading to squamous metaplasia. Many inflammatory bronchopulmonary diseases are characterized by a bronchial epithelium remodeling[68] and by a progressive and altered ability of the airway epithelium to regenerate a normal and functional epithelium.[69] Chronic obstructive pulmonary diseases are also characterized by a permanent inflammatory context. The α7 nAChR is also now known as an essential regulator of inflammation.[21,70] Its inactivation or down-expression may contribute to the excessive proinflammatory pathway, the increased fragility of the airway epithelium and the altered epithelium

regeneration observed in COPD. Most patients with COPD have a history of chronic smoking. In these patients, α7 nAChR inactivation may results from chronic exposure to nicotine. Indeed, the α7 nAChR has been shown to be particularly susceptible to inactivation[33–35] and over exposure of bronchial epithelium cells to nicotine *in vitro* produced an antagonist-like effect (7). Interestingly, $\alpha7^{-/-}$ mice and αBTX-exposed HAEC cultures present the same airway epithelium remodelings, basal cell hyperplasia, and squamous metaplasia, as those observed in these smoking COPD patients.

In summary, α7 nAChR appears to be a key regulator of the plasticity of the human airway epithelium in controlling basal cell proliferation. Our results also suggest that α7 nAChR may be involved in the basal cell differentiation process. These effects may be determinant in the airway epithelium remodeling associated to cigarette smoke as well as in the development of COPD and lung cancers.

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